Intraspecific Variation in the Purple Secretion of the California Sea Hare, *Aplysia californica* Cooper

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THE SEA HARE is probably best known because of the red to purple fluid secreted from the margin of its mantle. There is only a superficial resemblance between this secretion and that of the genus *Murex*, which is the source of the anciently famous dye, "Tyrian" or "Royal Purple." Aplysia purple, however, found no practical use, probably because of one important deficiency—it turns a dirty brown upon exposure to air and sunlight.

Our knowledge of the nature and composition of aplysia purple has been increased periodically since DeNegri (1876) presented a series of beautifully colored spectra. The most significant of the early papers is that of MacMunn (1899), who attempted an analysis but concluded that there was no reason to believe that aplysia purple was composed of more than a single component. Durrien and Turchini (1925) published a minor observation which, unfortunately, was recognized as the oldest literature on the subject by some later authors. Schreiber (1932) presented the results of an extensive study of aplysia purple. He divided the purple into two components, one of which he showed to be a substance similar to urobilin and the other its precursor substance, which is also pigmented but very unstable. Fontaine and Raffy (1936) made suggestions as to the possible source of the pigment molecules from the food of the sea hare. Lederer and Huttrer (1942) used chromatographic columns to separate the two components found in the pigment. Fox (1953) summarized the work of the three last-mentioned authors. Christomanos (1955) presented a summary of biochemical studies on the purple of a questionable species he specified as *A. depilans*. He also reported isolating a green band on his powdered sugar column from the purple of the *Aplysia* which he refers to as *A. depilans*. He was unable to obtain sufficient concentrations to produce a spectrum.

The present writer noticed that there was a gross color difference between young animals and adults of the west coast sea hare, *Aplysia californica* Cooper, the younger producing a bluer, the older a more reddish secretion. Experiments to test the cause of this phenomenon led to more basic studies of the composition and instability of aplysia purple.

MATERIALS AND METHODS

To obtain aplysia purple uncontaminated by salt water, specimens of *Aplysia californica* Cooper at Lunada Bay, Palos Verdes, California, were caught and the mantle area was rinsed with distilled water. This usually caused sufficient irritation to stimulate flow of the secretion but not enough to cause the opaline gland to discharge its contaminating viscous protein material. The aplysia purple was caught in small vials, to each of which a

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drop of hydrochloric acid was added to prevent degeneration. The material was returned to the laboratory where it was refrigerated or frozen for long-term storage. Spectrophotometric curves and chromatographic separations were made using 10 per cent hydrochloric acid as diluent. Spectrophotometric curves were run on a Beckman Model DU spectrophotometer.

The writer used two methods of analysis. Chromatographic columns were prepared, using corn starch packed in a column two feet deep, in four-foot sections of soft glass tubing (⅜ in., outside diam.). A very small piece of glass wool at each end of the column held the corn starch in place.

The tube was placed in a stopper which in turn fitted into a suction flask. Approximately one half cc. of aplysia purple was placed in the top of this column. As soon as the substance had penetrated the column, the top of the tube was filled with 10 per cent hydrochloric acid and suction was applied to the base of the column. About 8 hours later, when pigment had reached the bottom, the tube was removed and cut into sections corresponding with the color components. The starch was removed and the color components were eluted with dilute hydrochloric acid. Instead of cutting up the column, suction can be continued and each fraction can be caught as it drips from the bottom of the column. This method is very slow, requiring several days for one complete separation.

It was found that quicker determinations, especially where it was unnecessary to collect the fractions, could be made by paper chromatography. Several alterations in the methods were used, depending upon the nature and purpose of the particular investigation being carried on. Square sections of Whatman No. 1 filter paper measuring 18 × 18 cm. were spotted or lined with aplysia purple at a point 1½ cm. from the margin which would become the base. The liquid was applied with either a fine pipette or with an Esterbrook No. 2 drawlet pen. Each paper was then folded twice parallel to the line of travel of the components, after which the folds were relaxed enough so that the papers would stand without any two parts of the paper being in mutual contact. The papers were stood in petri dishes containing about ½ cm. of dilute hydrochloric acid and each paper and its petri dish was then covered with a bell jar. Separations made by this method were complete in one hour. Great care was necessary, however, in making the original spot or line. Thin lines and small spots produced the best results, especially where the distance of upward migration was limited. Strips 3 feet long were used with large initial wide lines of pigment. These resulted in excellent separations but required from 8 to 12 hours. The papers were dried and stored until the time of elution. Lining rather than spotting simplified the separation and subsequent elution. The papers were cut into strips parallel with the original pigment line so that each strip contained only one color component. Elution was then made by "siphoning" dilute hydrochloric acid lengthwise through the strips thus formed.

EXPERIMENTAL DATA

To determine the spectrophotometric differences between the purple secretion of young specimens whose purple appears bluish and that of adult specimens which is of a reddish hue, studies were made upon diluted aplysia purple. The most striking differences, as shown in Figure 1, were: (1) the differences in relative height of the two peaks, and (2) the variation of 15 mμ in the location of the broad peak in the high 500 region.

The components of the purple of *A. californica* were separated in quantity by column chromatography. Though no blue region was distinct as a separate band on the starch column, the collected fraction from the extreme trailing region of the lavender zone gave high concentrations showing a deep blue gross color, which produced a different absorption curve than that of the earlier lavender collec-

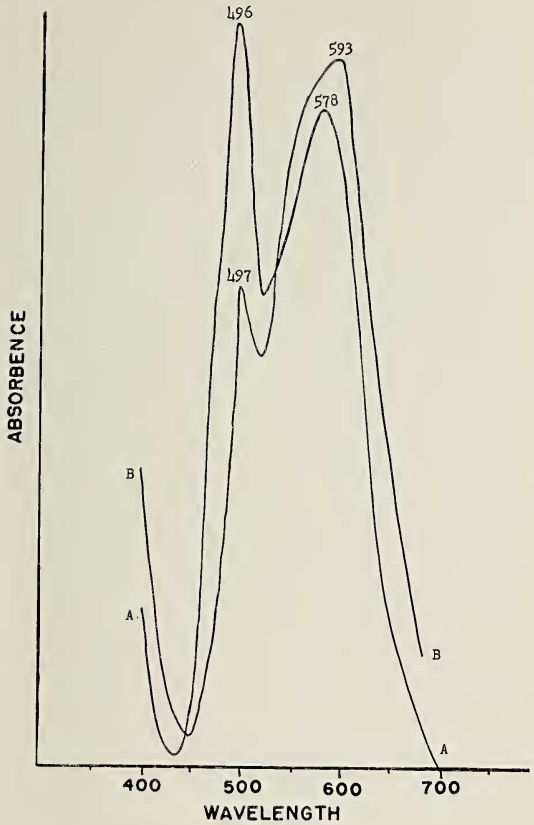

FIG. 1. The spectral curves of the aplysia purple taken from (A) a *Plocamium*-feeding adult, and (B) a young specimen showing no signs of feeding on *Plocamium*. The pigment was diluted in 10 per cent hydrochloric acid.

tions. The absorption spectrum (Fig. 2) showed a far sharper peak but a much less acute slope toward the red end of the spectrum than was noted earlier in the lavender collections. This suggested that it constituted a separate component. Paper chromatography was then tried as a means of separation, and by this it was possible to prove the existence of a third component of the aplysia ink of *A. californica*. On the dried paper a rhodonite pink component (Ridgway, 1912) left a wide band in the center of the paper. The second, a deep lavender, lagged behind at one-quarter the distance above the starting line. Scarcely clearing the initial line was a calamine blue area. Elution produced weak concentrations of the pigments, but these were adequate to produce curves on the Beckman Model DU spectrophotometer. However, these papers were dried and stored before elution, and this treatment gave opportunity for oxidative changes (Fig. 2).

DISCUSSION AND CONCLUSIONS

Though a component was isolated which gave a virtually pure curve at 496 mμ (Fig. 3), the writer has never succeeded in isolating a fractional component with a peak in the high 500 region which did not also have a weak peak at 499.5 mμ (not 496). As this material having two peaks (which will be shown to be composed of more than one, separable, but similar components) stands under refrigeration over a period of time, the relative heights of the two peaks change. The D band peak decreases and the F band peak increases in height, with a gradual shift of the F band from 499.5 to 497 and finally to 496 mμ, eventually giving approximately the same spectrum and gross color as the original aplysia purple from which it was separated (Fig. 3).

Aplysia purple is believed to be a bilin (Fox, 1953), a linear chain of pyrrole molecules connected by –CH= or $-CH_2-$ linkages, as it gives a positive Gmelin reaction. Brode (1955), by using commercial dyes, showed that the spectrophotometric effect of these linkages is insulating for $-CH_2-$ and coupling for the –CH= configaration. In *Aplysia* the pyrrole molecules of the bilins seem to be joined by the insulating linkages when in the blue state, which thus inhibits the normal F band absorption characteristic of the chain of pyrrole molecules absorbing together. In time, these insulating linkages gradually change to coupling linkages, perhaps due to enzymatic activity or other causes as yet unknown. As Brode (1955) puts it, "If the linkage is changed to a conjugating (=coupling) connection . . . there is a marked change in the spectrum with the production of a single resonance structure involving both

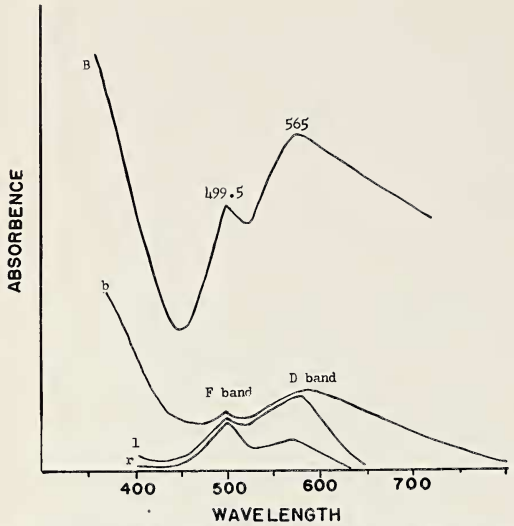

FIG. 2. Curve B shows the extreme trailing blue portion of the column chromatogram. The paper chromatographic curves are indicated by small letters. The curves of the rhodanite pink (r), the lavender (l), and the calamine blue (b) regions were read from weak solutions produced by eluting dried papers.

chromophores as a single coplaner chromophore." The curves he used as examples compare markedly with the curves of aplysia purple.

The writer has not yet found a satisfactory explanation for the instability of the D band absorption peak, which seems quite sporadic in its variation but doubtless follows well-defined laws.

Schreiber's work (1932) on the Mediterranean purple-producing *Aplysia* indicated that the peak in the F band (at approximately 500 mμ) represents the absorption by a substance similar to urobilin, and that the other peak in the high 500 region (Schreiber's broad unstable D band) represented the precursor of this substance. The above data confirm his theory.

It seems that the fast-growing young do not possess as much of the red finished product as do the adult specimens and hence secrete a larger percentage of the blue precursor substance. No basic differences other than that of relative quantity of the substance and its precursor are indicated by the spectrophotometric studies.

The condition of the individual animal is indicated as being highly influential in this respect as well. Well-fed adult specimens seem to have an excess of purple which is secreted at the slightest provocation, and often with no provocation at all.

The vertebrate bilins are derived from the breakdown of the tetrapyrrole molecules of hemoglobin. However, in plants tetrapyrrole molecules are the basis of chlorophyll. The quantities of aplysia purple produced would indicate a plentiful source such as chlorophyll, which is consumed in quantity by these algae eaters.

The bilichromes of the red algae may well be an additional rich source of the purple, as suggested by Fontaine and Raffy (1936). This is supported by the fact that adult animals feeding largely on *Plocamium pacificum*, which has a low chlorophyll but a high phycobilin content, usually produce a much greater quantity of pigment and with less provocation than those subsisting on other algae.

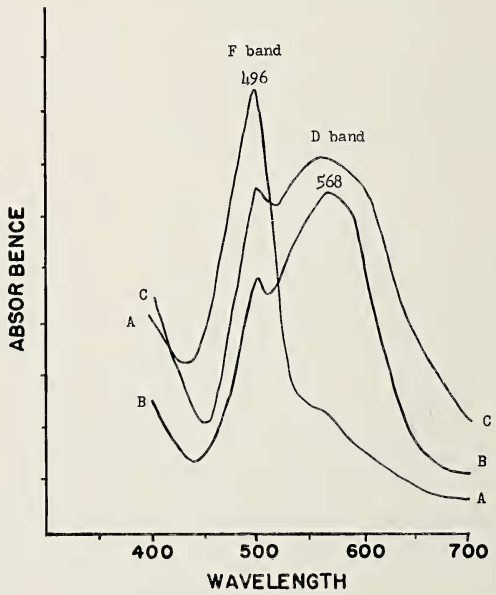

FIG. 3. The absorption spectra of the red (A), and composite lavender and blue (B) regions from the column separations. After the solution giving curve B had been in the refrigerator for two weeks it had changed its gross color and produced the curve marked C.

However, these other algae can not be the only source as the writer has kept small *A. californica* until they have approximately tripled in size while feeding them on parsley and celery tops, which contain none of the phycobilins, and he has found that they could still produce the purple secretion, though in reduced quantity.

Lederer and Huttrer (1942) followed current practice in naming the two components which they isolated from the purple of *A. punctata*. To the redder of the two components, which is the "urobilin" of Schreiber (1932), they gave the name *aplysiorhodin;* and to the bluer component, or the "urobilinogen" of Schreiber, they gave the name of *aplysioviolin*. For convenience of identification the writer gives the name *aplysioazurin* to the additional blue component he has isolated from the secretions of *A. californica*.

Spectrophotometrically, this new bluem coponent is responsible for the sharp rise in absorption below 425–450 mμ, as noted in all spectral curves produced by solutions from which it had not been removed. The rise was absent from the spectra of the other two components in the paper separations (Fig. 3).

Actually, in view of Schreiber's work (1932) aplysioviolin would be the precursor of aplysiorhodin. It is not yet evident what the role of aplysioazurin may be.

SUMMARY

From the foregoing discussion of aplysia purple the writer proposes: (1) that there are two apparent absorption peaks in the spectrum of aplysia purple from *Aplysia californica* when hydrochloric acid is used as the solvent; (2) that the relative height of these two peaks indicates the proportion of precursor to finished product in the purple from an individual specimen; (3) that the gross color varies widely from animal to animal; (4) that column chromatography as used here is superior for large scale separations but poor for qualitative separation; (5) that paper chromatography is more sensitive and is superior for quick qualitative isolation of components into individual zones; and (6) that the purple of *A. californica* contains a third component, *aplysioazurin*, in addition to the major components, *aplysiorhodin* and *aplysioviolin*.

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